Summary of Research

1) **Title of the Grant: Calcium/Calmodulin-mediated Gravitropic Response in Plants**

2) **Grant Number: NAG5-4841**

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5) **Period of Report: 1997-2001**

Summary of Research

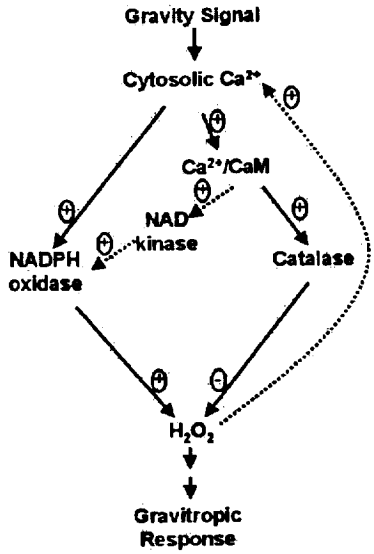
The goal of this project was to gain a fundamental understanding of how calcium/calmodulin-mediated signaling is involved in gravity signal transduction in plants. During the period of support, significant progress was made in elucidating the role of calmodulin and its target proteins in gravitropism. This laboratory has made breakthroughs by cloning and characterizing genes that are involved in calcium/calmodulin-mediated signaling. Some of these genes show altered expression under hypergravity and simulated microgravity conditions. A major advance was made in our attempts to understand gravity signal transduction by cloning and characterizing a catalase which requires calcium/calmodulin for its activation. Our results suggest that calcium/calmodulin have dual roles in regulating the level of hydrogen peroxide (H_2O_2), a signal molecule that plays a major role in gravitropism. It is well established that auxin plays a major role in gravitropism. Our results indicate that there is a "cross-talk" between calcium/calmodulin-mediated signaling and auxin-mediated signal transduction. Auxin-regulated SAUR proteins that are involved in gravitropism bind to calmodulin in a calcium-dependent manner. A novel chimeric calcium/calmodulin-dependent protein kinase was cloned and characterized and its role in gravity signal transduction was investigated. These studies have provided some answers to the fundamental questions about how signal molecules such as calcium, H_2O_2, and hormones such as auxin bring about the ultimate gravitropic response and the integral role of calmodulin in gravity signal transduction. This NASA-funded study has led to some spinoffs that have applications in solving agricultural problems. The Washington State University Research Foundation has obtained several patents related to this work.

Specific Accomplishments

1. Plant catalase is a Ca^{2+}/CaM-binding protein and it plays a role in gravitropism by controlling hydrogen peroxide homeostasis: Hydrogen peroxide (H_2O_2) is one of the toxic reactive oxygen species present in all aerobic organisms. Higher levels of H_2O_2 accumulation in the cell causes significant cell damage unless dealt with properly. Recent studies, however, also support that H_2O_2 acts as an intracellular signal to activate physiological responses to different stimuli. To allow for these different roles, cellular levels of H_2O_2 must be tightly controlled. Catalase is one of the major anti-oxidant enzymes which breaks down H_2O_2 into water and oxygen. By screening an *Arabidopsis* seedling cDNA expression library using ^{35}S-labeled CaM, catalase3 (AtCat3) was isolated and identified as a CaM-binding protein. The CaM-binding region was mapped (415 to 451 in the C-terminus) where high homology exists among plant catalases. However, there is no homology in this region between plant, bacterial and animal catalases, suggesting that CaM binds to plant catalases, but not to bacterial and animal catalases. To study the role of Ca^{2+}/CaM in regulating catalase activity, plant catalase was purified and used. In addition, the effects of Ca^{2+}/CaM in regulating human, bovine and *Aspergillus* catalase were studied. *In vitro* activity assays revealed that Ca^{2+}/CaM increased plant catalase activity by

~ 2.5 fold. Ca^{2+} or CaM alone had no effect on enzyme activity. Ca^{2+}/CaM did not activate human, bovine or *Aspergillus* catalases. Our results demonstrated that Ca^{2+}/CaM controls the breakdown of cellular H_2O_2 by binding to and activating plant catalases. Other reports suggest that Ca^{2+}/CaM may indirectly regulate NADPH oxidase leading to the production of H_2O_2 via modulation of NAD kinase activity. Based on these findings, we have proposed a model for a dual regulation of the level of cellular H_2O_2 by Ca^{2+}/CaM and the significance of this dual regulation in gravitropism.

Calcium is known to alter directional growth in plants and plays a critical role in gravitropism. To test the effect of H_2O_2 on gravitropic response in maize roots, 3-day-old seedlings were dipped in the H_2O_2 solution at different concentrations for varying periods. They were then rinsed in distilled water and the seedlings were arranged horizontally on the plain agar medium. 1 mM H_2O_2 treatment blocked the gravitropic bending. To test whether the interaction between Ca^{2+} signaling and H_2O_2 signaling exists, we studied root gravitropic responses after placing the agar block containing both 10 mM $CaCl_2$ and 10 mM H_2O_2 on the upper surface of the root tip. The agar plates were then arranged so that the roots were in a horizontal position. Surprisingly, the root oriented towards the gravity (downward) instead of towards the Ca^{2+} or H_2O_2 source. For controls, plain agar blocks were placed on the upper side of the root tips. Photographs were taken to study the root orientation. Our results suggest that there is an interrelationship between Ca^{2+}/CaM and H_2O_2, which triggers a series of events leading to the gravitropic response. A model describing the interaction between Ca^{2+}/CaM/H_2O_2 in triggering gravitropic response is shown below.



2. Evidence for the involvement of Ca^{2+}/CaM-mediated signaling in auxin-induced gravitropism: The use of ^{35}S-labeled CaM to screen a corn root cDNA expression library has led to the isolation of a CaM-binding protein, encoded by a cDNA with sequence similarity to

small auxin up RNAs (*SAURs)*, a class of early auxin-responsive genes. The cDNA designated as *ZmSAUR1* (*Zea mays SAURs*) was expressed in *E. coli* and the recombinant protein was purified by CaM affinity chromatography. The CaM-binding assay revealed that the recombinant protein binds to CaM in a Ca^{2+}-dependent manner. Deletion analysis revealed that the CaM-binding site was located at the N-terminal domain. A synthetic peptide of amino acids 20-45, corresponding to the potential CaM-binding region, was used for Ca^{2+}-dependent mobility shift assays. The synthetic peptide formed a stable complex with CaM only in the presence of Ca^{2+}. The CaM affinity assay indicated that ZmSAUR1 binds to CaM with high affinity (*Kd*, ~15 nM) in a Ca^{2+}-dependent manner. Comparison of the N-terminal portions of all the characterized SAURs revealed that they all contain a stretch of the basic α-amphiphilic helix similar to the CaM-binding region of ZmSAUR1. CaM binds to the two synthetic peptides from the N-terminal regions of *Arabidopsis* SAUR-AC1 and soybean 10A5, suggesting that this is a general phenomenon ~ all SAURs. Northern analysis was carried out using the total RNA isolated from auxin-treated corn coleoptile segments. The *ZmSAUR1* gene expression began within 10 min, increased rapidly between 10-60 min, and peaked around 60 min following 10 μM NAA (α-naphthaleneacetic acid) treatment. These results indicate that *ZmSAUR1* is an early auxin-responsive gene. The CaM antagonist, W-7, inhibited auxin-induced cell elongation, but not auxin-induced expression of *ZmSAUR1*. This suggests that Ca^{2+}/CaM do not regulate *ZmSAUR1* at the transcriptional level. CaM binding to ZmSAUR1 in a Ca^{2+}-dependent manner suggests that Ca^{2+}/CaM regulate ZmSAUR1 at the post-translational level. Our data provide the first direct evidence for the involvement of Ca^{2+}/CaM-mediated signaling in auxin-mediated signal transduction. There are more than 20 *SAURs* in the *Arabidopsis* genome. However, we observed that only five of them share high homology with *ZmSAUR1*. Since roots also respond to auxin, we expected that *SAURs* will be induced during root gravitropism.

3. Effects of hypergravity and simulated microgravity on Ca^{2+}/CaM-regulated genes: We have performed ground-based studies to provide evidence that Ca^{2+}/CaM play a central role in gravity signal transduction using facilities at the Dutch Experiment Support Center which specializes in acceleration research. We have studied the effects of hypergravity and simulated microgravity on genes that are involved in Ca^{2+}/CaM-mediated signaling. To study the effects of hypergravity on gene expression, plants were subjected to 10 x earth gravity for varying periods (5 hrs to 5 days) using the MidiCAR centrifuge. To study the effects of simulated microgravity on gene expression, the Random Positioning Machine (RPM) was used (ranging from 5 hrs to 5 days). To study these effects of simulated microgravity and hypergravity on the expression pattern of catalase, *SAURs* and *AtSRs*, RT-PCR analysis was performed using gene-specific primers, which were designed from the least conserved regions of each gene. The *Arabidopsis* actin 8 gene (*ACT8*) was used as a positive internal control. PCR primers for detection of *ACT8* mRNAs were 5'- ATGAAGATTAAGGTCGTGGC-3' and 5'-TCCGAGTTTGAAGAGGCTAC-3'. Total RNA from two week old entire seedlings was treated with RNase-free DNase (GIBCO-BRL). Both cDNA synthesis and PCR amplification were performed (25 ng total RNA) using gene specific primers (SuperScript One-Step RT-PCR, GIBCO-BRL). To maintain the amplification of the internal control and our genes within the exponential phase, the number of

PCR cycles was adjusted to 25 cycles for *ACT8*, catalase and SAUR and 35 cycles for all *AtSR* genes (see also Final Summary of Research for NAG5-11364). The amplified PCR products (9 µl) were electrophoresed on a 1.5 % (w/v) agarose gel, stained with ethidium bromide, and scanned using an image analyzer. Preliminary results revealed an altered expression pattern of these genes regulated by simulated microgravity and hypergravity (see Fig. 5). For example, among three catalases, only one (*AtCat1*) showed a positive response to simulated microgravity treatment. Among six *AtSRs*, only four (*AtSR1,2,5,6*) showed detectable changes. Interestingly, expression of the SAURs (*AtSAUR1,2*) was not clearly detected when seedlings were exposed to microgravity, suggesting that simulated microgravity has altered auxin redistribution/transport. The PI recently presented a report on some of the recent findings at the joint meeting of the European Space Agency (ESA) and the International Society for Gravitational Physiology (ISGP) meetings in Stockholm, Sweden in June 2002. A manuscript summarizing these results is currently in press.

Publications resulting from this support:

Ramachandiran, S., Takezawa, D., Wang, W., and Poovaiah, B.W. 1997. Functional domains of plant chimeric calcium/calmodulin-dependent protein kinase: regulation by autoinhibitory and visinin-like domains. J. Biochem. 121: 984-990.

Poovaiah, B.W., Wang, W., Takezawa, D., Narayanan, S. and An, G. 1997. Calcium-mediated signaling in plants: calmodulin and calcium/calmodulin-dependent protein kinase. J. Plant Biol. 40: 190-197.

Liu, Z., Xia, M. and Poovaiah, B.W. 1998. Chimeric calcium/calmodulin-dependent protein kinase in tobacco: differential regulation by calmodulin isoforms. Plant Mol. Biol. 38: 889-897.

Wang, W. and Poovaiah, B.W. 1999. Interaction of plant chimeric calcium/calmodulin-dependent protein kinase with a homolog of eukaryotic elongation factor-1α. J. Biol. Chem. 274:12001-12008.

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Sathyanarayanan, P.V., Cremo, C.R. and Poovaiah, B.W. 2000. Plant chimeric Ca^{2+}/calmodulin-dependent protein kinase: Role of the neural visinin-like domain in regulating autophosphorylation and calmodulin affinity. J. Biol. Chem. 39: 30417-30422.

Yang, T. and Poovaiah, B.W. 2000. *Arabidopsis* chloroplast chaperonin 10 is a calmodulin-binding protein. Biochem. Biophys. Res. Commun. 275: 601-607.

Sathyanarayanan, P.V., William F. Siems, Jeffrey P. Jones and B.W. Poovaiah. 2001. Calcium-stimulated autophosphorylation site of plant chimeric calcium/calmodulin dependent protein kinase. J. Biol. Chem. 276: 32940-32947.

Book Chapter:

Poovaiah, B.W., Wang, W. and Yang, T. 2001. Novel calcium/calmodulin-modulated proteins: chimeric protein kinase and small auxin up RNA protein. In: Signal Transduction in Plants: Current Advances, Eds. S.K. Sopory, R. Oelmuller and S.C. Maheshwari, Kluwer Academic/Plenum Publishers.

Published Abstracts:

Poovaiah, B.W., Wang, W., Ramachandiran, S., Sathyanarayanan, P. 1997. Functional domains of plant chimeric calcium/calmodulin-dependent protein kinase: regulation by autoinhibitory and visinin-like domains. ASGSB Bulletin 11: 114.

Poovaiah, B.W., Liu, Z., Wang, W. and Sathyanarayanan, P.V. 1998. Functional domains and the genomic structure of chimeric calcium/calmodulin-dependent protein kinase. Gravitational and Space Biology Bulletin 12 (1): 30.

Wang, W. and Poovaiah, B.W. 1999. Interaction of plant chimeric calcium/calmodulin-dependent protein kinase with a homolog of eukaryotic elongation factor-1α. Gravitational and Space Biology Bulletin 13 (1): 42.

Yang, T. and Poovaiah, B.W. 1999. Molecular evidence for the involvement of calcium/calmodulin in auxin action. Gravitational and Space Biology Bulletin 13 (1): 25.

Sathyanarayanan, P.V., Cremo, C.R., Siems, W.F. and Poovaiah, B.W. 2000. Chimeric calcium/calmodulin-dependent protein kinase: Role of the neural visinin-like domain in regulating autophosphorylation and calmodulin affinity. Gravitational and Space Biology Bulletin 14 (1): 16.

Poovaiah, B.W. and Yang, T. 2000. Gravitropism: Cross-talk between calcium/calmodulin and hormone mediated signaling. Gravitational and Space Biology Bulletin 14 (1): 46.

Patent received during this grant period:

Poovaiah, B.W., Liu, Z., Takezawa, D. and Patil, S. Compositions and methods for production of male sterile plants. Patent issued June 20, 2000. No. 6,077,991.
Assignee: Washington State University Research Foundation, Inc., Pullman, WA.